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Antimicrobial potential and cytotoxicity of endophytic fungi crude extracts from *Ricinus communis* of Tanzania

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Abstract. *Shemnkande NS, Lyantagaye SL, Mpenda FN. 2023. Antimicrobial potential and cytotoxicity of endophytic fungi crude extracts from* Ricinus communis *of Tanzania. Asian J Trop Biotechnol 20: 69-78.* The study aimed to assess the antimicrobial potential and cytotoxicity of crude extracts from endophytic fungi found in *Ricinus communis* L. from Tanzania. The researchers isolated and identified fifty-one fungi species from the leaves and roots of *R. communis*. The isolates were morphologically characterized, considering color, size, shape, elevation, margin, and density parameters. The isolates were further analyzed using Sanger sequencing and bioinformatics tools to determine their phylogenetic relationships and identify the isolates. Two techniques were deployed to evaluate the antimicrobial potential: disc diffusion and microdilution. The disc diffusion method measured the inhibition zones formed by the fungi extracts against selected bacterial strains (*Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. In comparison, the microdilution technique determined the crude extracts' Minimum Inhibitory Concentrations (MIC) against the bacterial strains. Cytotoxicity of the crude extracts was evaluated using the brine shrimp lethality assay; Brine shrimp larvae were exposed to the extracts to evaluate their potential toxicity. The study successfully isolated 51 fungi species from *R. communis* and observed significant morphological variation. Four endophytic fungi (*Penicillium menonorum, Curvularia verruculosa, Aspergillus niger* and *Aspergillus aculeatinus*) exhibited potent antibacterial activity against *B. subtilis, E. coli, P. aeruginosa,* and *S. aureus*, with low minimum inhibitory concentrations (3.125 µg/mL to 0.098 µg/mL). Cytotoxicity tests on brine shrimp larvae indicated the crude extracts' non-toxicity, suggesting their potential as safe therapeutic agents. The findings suggest that endophytic fungi from *R. communis* possess potential therapeutic applications against drug-resistant pathogens, warranting further investigation of their bioactive compounds and broader biotechnological uses.

Keywords: Antimicrobial activity, cytotoxicity, endophytes, *Ricinus communis*, Tanzania

INTRODUCTION

The United Nations targets 3.3 to end the epidemic and neglected tropical diseases (Fitzpatrick and Engels 2016; Bangert et al. 2017; Raviglione and Maher 2017). In developing countries, the majority of deaths, around 90%, are caused by various infectious diseases such as tuberculosis, malaria, and HIV/AIDS (Gavazzi et al. 2004). For instance, Tanzania reports approximately 18% and 15% of airborne and waterborne infections annually. Current control measures for infectious diseases heavily rely on antifungal, antimalarial, and antibacterial drugs (Fair and Tor 2014). However, these available control options are compounded by the emergence and reemergence of drug-resistant infectious agents, which entails searching for alternatives like exploring the untapped potential of medicinal plants and their related endophytes (Anand et al. 2019).

Plants are valuable sources of organic compounds, most of which possess medicinal properties (Anand et al. 2019). Secondary metabolites produced by plants, including coumarins, alkaloids, essential oils, flavonoids, lectin, phenolics, terpenoids, tannins, polypeptides, and acetogenins, are utilized in the synthesis of antibiotics for the curing of infectious ailments (Gurib-Fakim 2006; Gautam and Avasthi 2019). According to Elijah et al. (2020), medicinal plants are those whose components, such as leaves, seeds, stems, roots, and fruits, are used to treat various human and animal ailments. Approximately 80% of the global population relies on herbal medicines for medical care, with people in underdeveloped countries extensively using medicinal plants (Mahady 2005; Gurib-Fakim 2006). These plants control various illnesses, including cough, diarrhea, dysentery, malaria, smallpox, syphilis, taeniasis, cholera, tuberculosis, and fever (Rakotoarivelo et al. 2015).

Although it is not widely supported by empirical evidence, communities of Tanzania have long been using *Ricinus communis* L. leaves, seeds, and roots to cure microbial infections and non-infectious illnesses. However, in other regions of the world, *R. communis* is recognized for its potential in numerous areas, including antifertility, anticancer, antioxidant, antidiabetic, antiulcer, antimicrobial, anti-asthmatic, cytotoxic, anti-inflammatory, and wound healing effects (Elijah et al. 2020). Nevertheless, the direct harnessing of medicinal plants has environmental implications, and therefore, exploring alternative sources of secondary metabolites, such as tapping into the potential of endophytes, has garnered significant attention.

Endophytes are microorganisms living in the tissues of various plant parts, such as roots, stems, seeds, and leaves,

establishing a mutualistic relationship without causing disease symptoms (Marwat and Fazal-Ur-Rehman 2017). Endophytes inhabiting medicinal plants have been observed to produce secondary metabolites with biological activities akin to those generated by the host plants (Gautam et al. 2013; Gautam 2014; Gouda et al. 2016). For instance, the endophytic fungus *Diaporthe* sp. is isolated from the medicinal plant *Cinchona ledgeriana* (Howard) Bern.Moens ex Trimen has yielded compounds such as quinine, quinidine, and cinchonine; plants own secondary metabolites (Rahmawati et al. 2021). A similar pattern emerged with the endophytic fungus *Fusarium solani* from the *Camptotheca acuminate* Decne. produces the effective antineoplastic drug camptothecin, a compound also present in the host plant (Rahmawati et al. 2021). Another notable example is the identification of podophyllotoxin, a precursor for essential anticancer medications, not only derived from the medicinal plant *Podophyllum peltatum* L. Still, it has also within the endophytic fungus *Phialocephala fortinii* isolated from the same plant (Shah et al. 2021).

Based on these facts, it is likely that endophytes living in symbiotic association with *R. communis*, including those associated with the *R. communis* used by Tanzanian communities, may produce secondary metabolites with remarkable biological applications. Furthermore, the antimicrobial properties of endophytes found in *R. communis* used by Tanzanian communities have not yet been isolated, and the characterization of these endophytes from *R. communis* is yet to be conducted. Therefore, the present study aimed to investigate the chemical profile and biological activity of endophytes from *R. communis* found in Tanzania.

MATERIALS AND METHODS

Sample collection

Samples were collected from Lushoto District in the Tanga region located at 4° 57' 54.3168" S and 38° 30' 5.7132" E and around the University of Dar es Salaam, Tanzania main campus located at S 06°78'122" and E 039° 20'574". Visually healthy-looking leaves and roots of *R. communis* were carefully collected in sterile polyethylene bags and stored. Voucher samples, required for proper identification and documentation, were promptly delivered to a botanist at the Botany Department, University of Dar es Salaam, and given a voucher number NSS 01. Following the authentication of the voucher samples, the collected plant materials were transported for further processing to the Department of Molecular Biology and Biotechnology laboratory at the University of Dar es Salaam.

Isolation and morphological characterization of endophytic fungi

Endophytic fungi were isolated following the procedures outlined in the study conducted by Mwanga et al. (2019) with minor modifications. Samples (plant parts) were subjected to sterilization steps to eliminate any external contaminants. Tap water was used to remove debris on the plant parts and soil contaminants, followed by air drying. Next, samples were sequentially immersed in 70% ethanol for 3 minutes to ensure sterility and 0.4% NaOCl for 1 minute. Samples were rinsed thrice with sterile distilled water for 1 minute and gently dried using sterile tissue paper. Then, processed plant parts were cut into small segments of approximately 1 cm in length. Subsequently, 4 segments were placed in Petri dishes containing Potato Dextrose Agar (PDA) supplemented with chloramphenicol (250 mg/mL) to prevent bacterial growth. Petri dishes were then incubated in the dark at 30°C for 4-6 days. Regular observations were made to monitor the growth of fungal colonies. Fungi isolates were purified by cutting the tips of the growing fungal hyphae using a sterile blade. Next, transfer them to sterile Petri dishes containing PDA supplemented with 250 mg/mL chloramphenicol. The petri dishes were carefully sealed with cling film and incubated at room temperature for 5 days. Pure cultures of the endophytic fungi were obtained through serial sub-culturing. These cultures were maintained in glycerol at 4°C for consequent screening and analysis.

Morphological characterization

Morphological characterization of endophytic fungi isolates involves an examination of various parameters such as mycelia types, isolate color, size, elevation, density, and margin of the isolates. These observations were made to assess the visual characteristics of the fungal isolates.

Molecular characterization

The collection of fungal mycelia was performed according to the protocol outlined by Aboul-Maaty and Oraby (2019). Fungal mycelia were transferred from Potato Dextrose Agar (PDA) to Potato Dextrose Broth (PDB) in 250 mL Erlenmeyer flasks. The flasks containing mycelia were placed on a shaker and incubated at 28°C for four days to allow growth. After incubation, approximately 100 mg of mycelia were collected by centrifugation at 12,000 rpm for five minutes, followed by genomic DNA extraction. The DNA extraction was conducted using the CTAB method, as explained by Aboul-Maaty and Oraby (2019), with minor modifications. DNA concentration and purity were determined using a Nanodrop spectrophotometer (Nanodrop One, Thermo Scientific, USA) at 260/280nm absorbance.

Amplification of rDNA Internal Transcribed Spacer (ITS) regions of the isolates was employed through the PCR method as described by Martin and Rygiewicz (2005) using primer pairs ITS1 (5’-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5’-TCCTCCGCTTATTGATATGC-3'). Next, PCR products were run on agarose gel electrophoresis, and the gel was analyzed using a gel documentation system (Shiva Scientific Company).

Bioinformatics analysis

The PCR products of fungal isolates (four isolates, Table 5) that were selected following initial screening were Sanger sequenced at Macrogen Europe (Amsterdam Medical Center), and the sequences were analyzed by

bioinformatics tools as previously described by Jeewon et al. (2013). Geneious Bioinformatics software was then used to trim and generate consensus sequences. A similarity search was done using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI). Closely related sequences were retrieved from GenBank of NCBI, and Molecular Evolution Genetic Analysis (MEGA X) software was used on multiple sequence alignment using the MUSCLE algorithm. Then, using aligned sequences, a neighbor-joining phylogenetic tree was constructed using MEGA X to depict genetic relatedness between isolates found in the present study and the previous isolates retrieved from GenBank of NCBI.

Mass cultivation and endophytic fungi crude extract harvesting

The primary screening was conducted to identify endophytic fungi with antimicrobial activity. Of the 51 isolated endophytic fungi, only 4 endophytes exhibited antimicrobial activity. The 4 fungal isolates were subsequently subjected to mass cultivation to produce metabolites. Media and mycelia for mass cultivation were prepared following the protocol outlined in the study conducted by Martin and Rygiewicz (2005). Briefly, mycelia on PDA agar were cut using a sterile blade and then placed into multiple 1,000 mL conical flasks containing 500 mL of sterile potato dextrose broth media to increase the potential of higher production of secondary metabolites. Subsequently, the flasks were incubated at room temperature with regular shaking for 4 weeks. After 4 weeks of incubation period, the cultures of endophytic fungi were filtered to eliminate the mycelia mats using cotton gauze. A solvent extraction procedure was employed to prepare crude extracts (Hajrah et al. 2018). Equal filtrate and ethyl acetate volumes were carefully measured and placed in a separating funnel. The mixture was shaken vigorously for 10 minutes and then left to settle, allowing the separation of cell masses from the solution. The aqueous solution was discarded, and a sterilized flask collected the organic solution. Any excess solvent was removed using a rotary vacuum evaporator (BUCHI Rota vapor Model R-210) at a temperature of 45°C under reduced pressure. The resulting fungal crude extracts were weighed, dissolved in Dimethyl Sulfoxide (DMSO), and stored for subsequent antimicrobial assay at 4°C.

Antimicrobial activity screening of crude extracts

With some modifications, antimicrobial activity was performed through the disc diffusion method, as explained by Mwanga et al. (2019). Inoculums of test organisms (Table 1) in normal saline were prepared from an overnight nutrient agar bacterial culture based on 0.5 McFarland standards (approximately 1.5×10^8 CFU/mL). Six mm diameter sterile Whatman discs were soaked with 10 µL of each crude extract, and the crude extracts were at the concentration of 200 mg/mL. After soaking, the discs were allowed to dry, placed on Mueller-Hinton agar plates, and then pre-inoculated with test microorganisms (bacteria, Table 1).

Table 1. List of selected test microorganisms for antimicrobial activity screening

Microorganism name	Culture number	Gram's reaction
Staphylococcus aureus	ATCC 29213	Gram-positive
Escherichia coli	ATCC 8736	Gram-negative
Pseudomonas aeruginosa	ATCC 6539	Gram-negative
Bacillus subtilis	ATCC 6051	Gram-positive

The control experiments followed the same procedures, but gentamicin and Dimethyl Sulfoxide (DMSO) were used as positive and negative controls instead of crude extracts; an incubation was followed on plates at 37°C for 24 hours. The growth inhibition zones after incubation were measured and recorded. Notably, all the antimicrobial test experiment results were presented as the (mean ± standard deviation) and performed in duplicate.

Minimum Inhibitory Concentration (MIC) testing

Minimum Inhibitory Concentrations (MICs) were determined by the microdilution method using 96-well microtitre plates (Lema et al. 2022). Initially, the plates were pre-loaded with 50 µL of Muller-Hinton broth media in each well; next, to make a total volume of 100 µL in the first wells, followed by the addition of 50 µL of the fungal crude extracts (100 mg/mL) into the first wells of each row tested. After thoroughly mixing, 50 µL was drawn from each well of the first row and put into wells of the next row. Then, to the last wells at the bottom, the process was repeated down the columns, where 50 µL was discarded. After that, 50 µL of the bacterial and fungal suspension 0.5 McFarland standards (approximately 1.5×10^8 CFU/mL) was added to make the final volume of 100 µL in each well. Rows containing gentamicin (50-0.024 µg/mL) were used as a positive drug, while Dimethyl Sulphoxide (DMSO) was used as a negative control. Plates were then incubated for 24 hours at 37°C. Each extract's MIC was determined by adding 30 µL of 0.05% p-Iodonitrotetrazolium (INT) chloride in each well, followed by incubation for 30 minutes bacteria; a color change in pink indicated bacterial growth. The lowest concentration, which showed no bacterial growth, was considered MIC.

Cytotoxicity assay

Brine Shrimp Lethality Assay (BSLA) was utilized to assess the presence of bioactive compounds in the extracts and evaluate their toxicity, following the protocol described by Nondo et al. (2011). Initially, 40 mg of each crude extract was accurately measured and dissolved in 1 mL of Dimethyl Sulfoxide (DMSO), resulting in a 40 mg/mL stock solution. For the brine shrimp hatching, 3.8 g of sea salt was measured and dissolved in 1 L of distilled water, creating a media solution with a concentration of 3.8 g/L. A tank was prepared, divided into two compartments by a perforated polythene wall. One part of the tank, covered by the wall, was filled with 1 L of the media solution, and 0.5 g of brine shrimp eggs were added. The left was uncovered, and the other part of the tank was illuminated with a lamp.

The tank was then left undisturbed for 36-48 hours to allow for the hatching of brine shrimp eggs.

After hatching, different concentrations of the extract were prepared. Varying volumes were drawn from the stock solution to achieve concentrations of 240, 120, 80, 40, 24, and 8 µg/mL. Each concentration was added to separate vials, with each vial containing 10 brine shrimp larvae. The volume in each vial was adjusted to 5 mL using artificial seawater prepared in 1 L of distilled water by dissolving 3.8 g of sea salt. Each concentration was tested in duplicate. The negative control consisted of vials containing brine shrimp larvae, artificial seawater, and DMSO only. The vials were then incubated under light for 24 hours. Following incubation, the number of dead larvae in each vial was counted, and the mean mortality was calculated.

Statistical analysis

Moreover, R software (4.3.0 version) was used to perform descriptive and inferential statistics. Next, the Shapiro-Wilk test was used to determine the normality of the inhibition zone and MIC data, and it found that data were not normally distributed. Therefore, the Kruskal-Wallis test was used to determine significant differences between the data. In addition, the correlation between the inhibition zone and MIC data was determined using Spearman rank correlation, with the significant difference set at $P<0.05$ levels.

RESULTS AND DISCUSSION

Abundance and morphological appearance of endophytic fungi from *R. communis*

Moreover, 51 endophytic fungi were isolated from the leaves and roots of *R. communis*. Therefore, 29 isolates were from roots, while 22 were from leaves. Figure 1 depicts the morphological appearance of 4 endophytes selected after initial screening for antimicrobial activity.

Fungi isolates were characterized based on their morphological features. There was a significant variation among the fungal isolates regarding color, size, shape, elevation, margin, and density (Table 2).

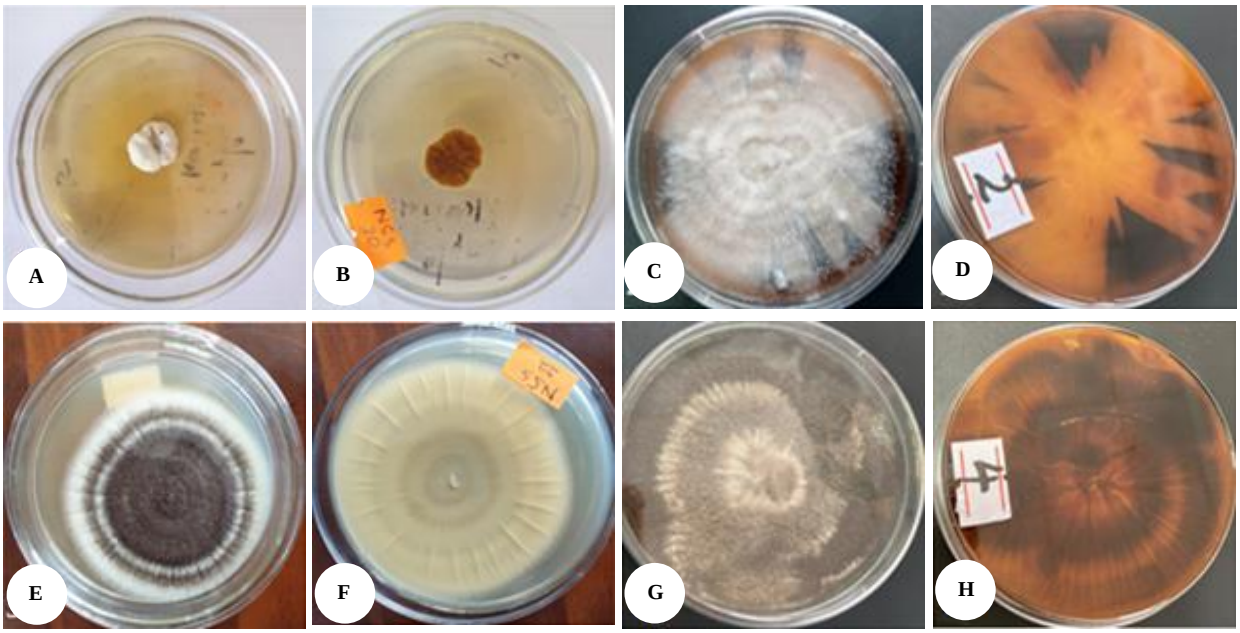

Figure 1. In the image, A and B are front and back views of *Penicillium menonorum* (MT529969.1), C and D are front and back views of *Curvularia verruculosa* (MF568070.1), E and F are front and back view of *Aspergillus niger* (LC496501.1), G and H are front and back view of *Aspergillus aculeautinus* (MT023714.1)

Table 2. Growth and morphological characteristics of endophytic fungi isolated from *Ricinus communis*

Isolate ID	Color		Size (mm)	Shape	Elevation	Margin	Density
	Front view	Back view					
1	Whitish	Brownish	23	Irregular	Raised	Undulate	Dense
2	whitish brownish	brownish blackish	50	Circular	Flat	Filamentous	Medium
3	whitish blackish	Grayish	57	Circular with margins	Flat	Filamentous	Dense
4	whitish blackish	Brownish blackish	60	Circular	Flat	Filamentous	Dense

Molecular identification of endophytic fungi from *R. communis*

The 51 isolates were confirmed to be fungal endophytes by PCR amplification of genomic DNA using ITS 1 and ITS 4 fungal-specific primers. As expected, the amplicon size was around 650 bp (Figure 2). The amplicon size ranged from 500 bp to 700 bp using ITS 1 and ITS 4 specific primers.

Furthermore, 4 isolates *Penicillium menonorum* (MT529969.1), *Curvularia verruculosa* (MF568070.1), *Aspergillus niger* (LC496501.1) and *Aspergillus aculeautinus* Noonim, Frisvad, Varga & Samson (MT023714.1) found in Figure 1, which were mass cultivated for fully antimicrobial test, were characterized by Sanger sequencing of PCR products of ITS 1 and ITS 4. Specifically, the GenBank database sequences were compared with the resulting sequences using the BLAST tool on NCBI. The analysis revealed that all the endophytic fungal sequences had similarity levels greater than 90% compared to the database sequences (Table 3). Also, the phylogenetic tree (Figure 3) displayed the molecular relatedness of fungal isolates in the present study. The four endophytes were of the genera *Penicillium, Aspergillus*, and *Curvularia.*

Antimicrobial activity of fungi crude extracts

Based on the initial screening observation, 4 out of 51 isolates were selected for mass cultivation and harvesting crude extracts, which were then tested for antimicrobial.

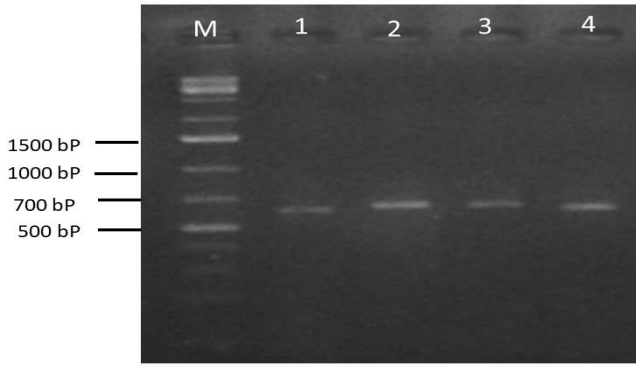

Figure 2. A gel picture of amplified DNA samples. M represents 1kb Plus DNA ladder (*New England Biolabs*), lane 1: *Pennicillum menonorum* (MT529969.1), lane 2: *Curvularia verruculosa* (MF568070.1), lane 3: *Aspergillus niger* (LC496501.1) and lane 4: *Aspergillus aculeautinus* (MT023714.1)

activity against selected organisms (Table 1) obtained in the Microbiology Laboratory. The inhibition zone was measured to provide insights into antimicrobial effectiveness against tested organisms. Interestingly, except for the crude extract of *A. niger* (LC496501.1), the overall antimicrobial activity of crude extracts was promising (Figure 3). The highest inhibition zone was 32.5 mm for the crude extract of *C. verruculosa* (MF568070.1) and *A. aculeautinus* (MT023714.1) against *B. subtilis*. In contrast, the crude extract of *A. niger* (LC496501.1) exhibited relatively weaker ($P<0.05$) antimicrobial activity against all tested organisms. For example, the lowest antimicrobial activity was 8 mm for crude extract of *A. niger* (LC496501.1) against *S. aureus*.

Table 3. Identified fungi and the percentage identity of isolated endophytic fungi from *R. communis* and that of the taxa found in the NCBI

Isolate	Name of species	Accession number	% identity
1	*Penicillium menonorum*	MT529969.1	100.00
	Penicillium menonorum	MT529566.1	99.83
	Penicillium pimiteouiense	MH045589.1	99.66
	Penicillium pimiteouiense	MH045584.1	99.66
	Penicillium pimiteouiense	MH029828.1	99.49
	Penicillium pimiteouiense	OQ874536.1	99.32
2	*Curvularia verruculosa*	MF568070.1	100.00
	Curvularia verruculosa	MH375716.1	99.45
	Curvularia verruculosa	OP412958.1	99.44
	Exserohilum rostratum	MN599601.1	99.07
	Curvularia americana	OP526931.1	99.07
	Curvularia verruculosa	OP412957.1	99.81
3	*Aspergillus niger*	LC496501.1	100.00
	Aspergillus niger	MT675916.1	97.77
	Aspergillus niger	LC496503.1	93.34
	Aspergillus niger	LC496502.1	92.73
	Aspergillus niger	MN593010.1	92.72
	Aspergillus niger	KU171053.1	97.42
4	*Aspergillus aculeatinus*	MT023714.1	100.00
	Aspergillus sp.	OQ913870.1	98.79
	Aspergillus aculeatinus	MK392046.1	98.95
	Aspergillus niger	FJ037755.1	99.64
	Aspergillus sp.	MT645654.1	98.78
	Aspergillus japonicas	OM096161.1	98.78

Note: % identity: percentage identity. Species with 100% identity are isolates from *Ricinus communis* of the present study

Table 4. Antimicrobial activities of the crude extracts (200 mg/mL) of endophytic fungi isolated from *Ricinus communis* against the pathogen of medical importance

Isolate	Test organisms			
	E. coli	***B. subtilis***	***S. aureus***	***P. aeruginosa***
Penicillium menonorum (MT529969.1)	25.0±0.71	31.5±0.71	28.0±0.41	31.5±0.71
Curvularia verruculosa (MF568070.1)	23.0±0.41	32.5±0.71	25.5±0.71	31.0±0.00
Aspergillus niger (LC496501.1)	12.0±0.41	15.0±0.00	8.0±0.41	10.0±0.00
Aspergillus aculeautinus (MT023714.1)	24.5±0.71	32.5±0.71	24.5±0.71	31.0±0.00
Positive control	27.0±0.00	25.0±0.00	26.0±0.00	25.0±0.00

Note: Positive control: Gentamicin (4 mg/mL); Numbers in the table are zones of inhibition in mm expressed as mean ± standard deviation

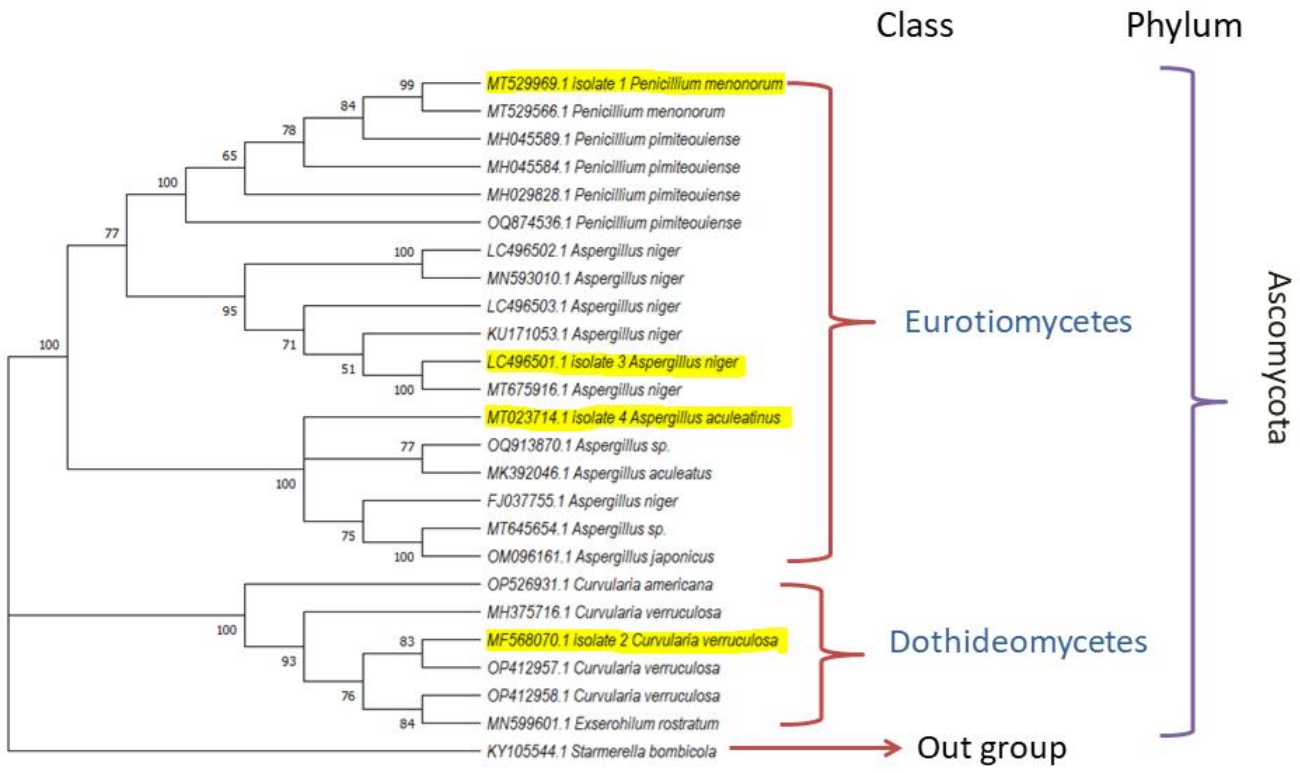

Figure 3. A Neighbor-Joining phylogenetic tree depicting the relationship of *Pennicillum menonorum* (MT529969.1), *Curvularia verruculosa* (MF568070.1), *Aspergillus niger* (LC496501.1), and *A. aculeautinus* (MT023714.1) with other related fungi found in the NCBI. The *Starmerella bombicola* C.A.Rosa & Lachance (KY1055441) was used as the outgroup. Branches represent values based on 1000 replications of Felsenstein's bootstrap method

Additionally, the crude extracts *P. menonorum* (MT529969.1), *C. verruculosa* (MF568070.1), and *A. aculeautinus* (MT023714.1) displayed a comparable antimicrobial activity pattern against all the organisms tested (Figure 4). When arranged in descending order, the test organisms exhibited varying levels of susceptibility to the crude extracts, with *B. subtilis* being the most susceptible, followed by *P. aeruginosa*, *S. aureus*, and *E. coli*. However, this pattern was not observed for crude extract 3 (*A. niger*, LC496501.1), as illustrated in Figure 4. In the bar graph in Figure 4, for example, the zone of inhibition of *A. niger* crude extract against *E. coli* was higher compared to that of *P. aeruginosa*, which was not the general trend for the rest of the crude extracts.

Minimum inhibitory concentrations

The Minimum Inhibitory Concentration (MIC) values of crude extracts from *R. communis* were determined against four test organisms, as shown in Table 5. Regarding Figure 5, among all crude extracts, the crude extract of *P. menonorum* (MT529969.1) exhibited the lowest MIC value against *B. subtilis*. Both crude extract of *P. menonorum* (MT529969.1) and crude extract of *A. niger* (LC496501.1) had the highest MIC values when tested against *E. coli* and *S. aureus*. The crude extracts of *P. menonorum* (MT529969.1), *C. verruculosa* (MF568070.1), and *A. aculeautinus* (MT023714.1) exhibited the same MIC value against *P. aeruginosa*. Crude extracts of *P. menonorum* (MT529969.1), *C. verruculosa* (MF568070.1), and *A. niger* (LC496501.1) displayed similar MIC values when tested against *S. aureus*.

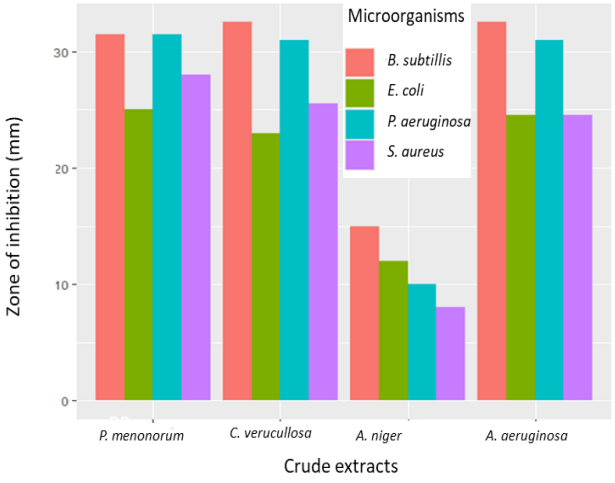

Figure 4. Antimicrobial activity pattern of endophytic fungi crude extracts isolated from *Ricinus communis* against tested organism based on inhibition zone

Table 5. Minimum Inhibitory concentrations (mg/mL) of four endophytic fungi ethyl-acetate crude extracts from *Ricinus communis* against tested microorganisms

Isolate	Test microorganism			
	E. coli	***B. subtilis***	***S. aureus***	***P. aeruginosa***
Penicillium menonorum (MT529969.1)	3.125	0.195	1.563	1.563
Curvularia verruculosa (MF568070.1)	0.781	0.781	1.563	1.563
Aspergillus niger (LC496501.1)	1.563	1.563	3.125	0.781
Aspergillus aculeautinus (MT023714.1)	1.563	0.391	0.781	1.563
Positive control	0.098	0.098	0.098	0.098

Note: gentamicin was used as a positive control

Table 6. Cytotoxic activities of endophytic fungi crude extracts from *Ricinus communis*

Isolate	LC_{50} (µg/mL)	95%CL	Regression equation	R^2
Penicillium menonorum (MT529969.1)	1011.58	214-513	y=27.345logx-32.178	0.854
Curvularia verruculosa (MF568070.1)	382.82	94-107	y=32.832logx-34.811	0.936
Aspergillus niger (LC496501.1)	170.88	46-625	y=44.926logx-50.307	0.863
Aspergillus aculeautinus (MT023714.1)	382.82	94-107	y=32.832logx-34.811	0.936

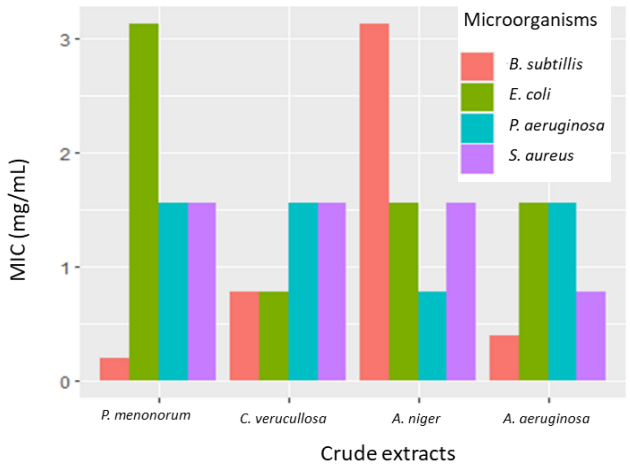

Figure 5. Antimicrobial activity pattern of endophytic fungi isolated from *Ricinus communis* against tested organism based on minimum inhibitory concentration

Cytotoxicity assay

All crude extracts from endophytic fungi of *R. communis* were not toxic to brine shrimp larvae since LC_{50} values for all extracts were above 100 µg/mL, as illustrated in Table 6.

Discussion

Due to an expected global challenge of antimicrobial resistance to most antibiotics currently in use (Cassell and Mekalanos 2001), there is an endeavor to search for novel drug structures or compounds from various sources, including plants (Anand et al. 2019). Although plants have been a good source of various anti-pathogenic agents (Manandhar et al. 2019), the direct harnessing of plants has environmental implications (Mehta et al. 2022). Therefore, alternative options like bio-prospecting of endophytes are of great interest. This is based on the ground that endophytes inhabiting medicinal plants have been found to produce secondary metabolites with biological activities similar to those produced by the host plants (van Wyk and Prinsloo 2018). The present study hypothesized that endophytes living in symbiotic association with *R. communis*, including those associated with the *R. communis* used by Tanzanian communities, may produce secondary metabolites with remarkable biological applications. Interestingly, the present study's findings supported the hypothesis because ethyl acetate crude extracts of endophytes isolated from *R. communis* had outstanding antimicrobial activity, as displayed in Table 4. Most importantly, the MIC for most extracts was very low, and the extracts did not demonstrate cytotoxicity upon BSLA assay.

This study represents the first report of isolation of endophytic fungi from *R. communis* of Tanzania. In this study, 51 endophytic fungi were isolated from the leaves and roots of *R. communis*. Morphological characterizations were employed in the identification of endophytic fungi isolates. However, there was a significant variation among the fungal isolates regarding color, size, shape, elevation, margin, and density, as depicted in Table 2. This may be due to genetic variability among the fungal isolates and different stages of fungal growth and development (Hallmann et al. 2007). Fungi isolates can exhibit considerable morphological diversity, and these variations provide valuable information for characterizing and distinguishing between different isolates (Hallmann et al. 2007). Based on the phylogenetic relationship (Figure 3), the isolates were found to be in 4 species (*P. menonorum*, *C. verruculosa*, *A. niger* and *A. aculeatinus*), three genera (*Penicillium*, *Curvularia*, and *Aspergillus*) and two classes (Dothideomycetes, Eurotiomycetes) under the phylum Ascomycota. The isolation of the four species in the present study is not surprising because the species have been isolated from other plants elsewhere. For example, in

the study conducted in Pudukkottai South India, *C. verruculosa* was isolated from the leaves of *Catharanthus roseus* (L.) G.Don (Parthasarathy et al. 2020). Furthermore, Díaz et al. (2019) isolated *A. niger* from rotten wood in the subtropical rainforest of Misiones, Argentina. The *P. menonorum* was also reported to be isolated from rhizosphere soil in Korea by Babu et al. (2015). Then, *A. aculeatinus* was isolated from the soil sample in Malaysia (Aziz and Zainol 2018). In addition, most of the endophytes are members of the phylum Ascomycota. Taken as an example in a study conducted by Abdulwehab et al. (2015), from different soil crust microhabitats and rhizosphere soils around the native bunchgrass in the USA, five Ascomycota fungi were isolated.

The crude extract of *A. niger* showed the lowest zone of inhibition (8 mm) when tested against *Staphylococcus aureus*. This may be caused by resistance of *S. aureus* towards crude extracts of *A. niger*. Similar to our results, Amina et al. (2017) conducted a study on the antibacterial activity of *Aspergillus* species; they reported that *A. niger* had a 7.33 mm zone of inhibition against *S. aureus*. Furthermore, crude extracts of *P. menonorum* (MT529969.1), *C. verruculosa* (MF568070.1), and *A. aculeatinus* (MT023714.1) displayed a comparable antimicrobial activity pattern against all the microorganisms tested. This observation is challenging; however, it may be explained by the adaptability of the fungi species to the host plant (*R. communis).* The observed trend has been reported in a previous study by Idris et al. (2013), which indicated that *Aspergillus* species and *Curvularia lunata* (Wakker) Boedijn had comparable inhibition zones against tested microorganisms.

The lowest MIC value obtained in the present study was 0.195 µg/mL for *P. menonorum* against Gram-positive bacterium *B. subtilis*, indicating that crude extracts of *P. menonorum* were most active against *B. subtilis.* Comparison of this observation with the previous results is difficult with two-fold explanations. First, *P. menonorum* is a novel species that has not been extensively studied, and such information is limited. Second, the inoculum of *P. menonorum* isolated from soil samples in Korea was evaluated for plant growth promotion (Babu et al. 2015), and therefore it is challenging to compare. However, the present information is very interesting as it demonstrates the wide biotechnological application of the species (*P. menonorum)* from medicine to agriculture applications.

The MIC value of *A. niger* was higher than *B. subtilis* compared to other crude extracts (Table 5). The information indicates that *B. subtilis* was less susceptible to ethyl acetate extract of *A. niger*. This observation was unexpected, considering that the *B. subtilis* possesses a thicker peptidoglycan layer on its cell wall, allowing antibiotic permeability. However, this may be explained by the chemical nature of the extract, which may be less effective against *B. subtilis.* Several antibiotics are effective against Gram-negative bacteria but less effective against Gram-positive bacteria. For example, ciprofloxacin is effective against *Pseudomonas aeruginosa*, a Gram-negative bacteria, but less effective against *S. aureus*, a Gram-positive bacteria (Baggio and Ananda-Rajah 2021).

MIC results did not depend on whether the test microorganisms were Gram-positive or Gram-negative. For instance, the crude extract of *P. menonorum* had a high MIC value of 3.125 µg/mL against the Gram-negative bacterium *E. coli*, and the crude extract of *A. niger* had a high MIC value of 3.125 µg/mL against gram-positive bacterium *B. subtilis*. These results contrast the common understanding that Gram-negative bacteria have reduced antimicrobial potency compared to Gram-positive bacteria due to their morphological differences in cell walls (Mpenda and Mkangara 2022). Gram-negative bacteria possess an additional outer membrane comprising lipopolysaccharides and proteins, serving as a protective barrier and making them less susceptible to the penetration of antimicrobial agents. In contrast, Gram-positive bacteria possess a thicker peptidoglycan layer, making it easier to penetrate antimicrobial agents (Sosovele et al. 2012). Sosovele et al. (2012) reported that actinomyces crude extracts showed a high MIC value of 5 µg/mL against *P. aeruginosa* while *B. subtilis* has MIC value of 0.1563 µg/mL. This understanding may not always be accurate because crude extracts are effective in Gram-positive and Gram-negative. For example, Yimgang et al. (2022) studied the antimicrobial activity of endophytic fungi inhibiting Cameroonian *Annona muricata* L.; they reported most MIC values similar to Gram-positive and Gram-negative. Additionally, Njeru et al. (2015) conducted a study on the antimicrobial activity of the crude extracts of *Premna resinosa* (Hochst.) Schauer, a Kenyan traditional medicinal plant; they reported that the antibacterial activity was high and broad spectrum, inhibiting both Gram-positive and Gram-negative bacteria. Based on these observations, the present results may be attributed to some extracts possessing secondary metabolites, which are effective against Gram-positive and Gram-negative bacteria.

The non-toxic nature of the crude extracts from endophytic fungi of *R. communis* towards brine shrimp larvae is an interesting finding, as indicated by LC_{50} values above 100 µg/mL (Table 6). According to Hadiza et al. (2014), LC_{50} < 1.0 µg/mL is considered highly toxic, LC_{50} 1.0-10µg/mL is toxic, LC_{50} 1.0- 30µg/mL is moderately toxic, LC_{50} > 30 <100 µg/mL is considered as mildly toxic and LC_{50} > 100 µg/mL is considered as non-toxic. These results suggest that the tested crude extracts do not cause acute toxicity toward brine shrimp larvae. This is an encouraging finding, implying that these endophytic fungi crude extracts may have a favorable safety profile. Previous studies that reported bioactive compounds with no toxicity in endophytic fungi supported the absence of toxicity in the crude extracts. For instance, a study conducted by Tibuhwa (2017) evaluated the cytotoxicity of *Boletus bicolor* Raddi and found that the extracts were significantly non-toxic towards brine shrimp larvae. Additionally, the study conducted by (Sosovele et al. (2012) reported that all ethyl acetate extracts from *Streptomyces* strains were non-toxic. The lack of toxicity in the crude extracts from *R. communis* endophytic fungi indicates the presence of bioactive compounds that are safe for use in further research and development. These extracts

can be valuable for exploring and isolating pharmacologically active compounds with reduced effects.

To conclude, in the present study, findings demonstrate that crude extracts of endophytic fungi from *R. communis* have promising therapeutic potential as they were effective against *E. coli*, *B. subtilis*, *S. aureus*, and *P. aeruginosa*, which are currently reported to pose great public health challenges due to drug resistance. However, the crude extracts from the present study also did not exhibit any cytotoxicity against brine shrimp larvae. Further studies should investigate bioactive compounds responsible for observed antibacterial activities. Research should also be conducted on the antimicrobial activity of crude extract from *R. communis* endophytic fungi against resistant bacterial strains like Methicillin-Resistant *S. aureus* (MRSA) and Methicillin-Resistant *P. aeruginosa* (MRPA). Additionally, further research should be conducted to assess if crude extracts from endophytic fungi present in *R. communis* may have other biotechnological applications in medicine, industry, and agriculture.

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